Adaptation of candidate vaccine strains of South African Territories (SAT) type foot-and-mouth disease virus to suspension cell culture

by

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Declaration

I, Thando Veto, declare that the dissertation which I hereby submit for the degree MSc (Tropical Animal Health), in the Faculty of Veterinary Science at the University of Pretoria, is my work and has not previously been submitted by me for a degree at this or any other tertiary institution.

19 December 2023

Ethics statement

The author, whose name appears on the title page of this thesis, has obtained the required ethics approval/exemption for the research described in this work.

The author declares that they have observed the ethical standards required in terms of the University's Code of ethics for scholarly activities

ABSTRACT

African buffalo (*Syncerus caffer*) are efficient maintenance hosts of three serotypes of foot-and-mouth disease (FMD) virus in South Africa, namely the South African Territories (SAT) types 1-3 viruses. Of these, the SAT-1 and SAT-2 serotypes historically caused the most FMD outbreaks in the country, however, SAT-3 outbreaks have also occurred in recent years. Because buffaloes represent a permanent source of infection for cattle, control of the disease hinges on the separation of buffalo and cattle through fencing and the establishment of a protection zone and a surveillance zone between the infected buffalo population in the Kruger National Park and the FMD-free zone. As fences can, and have been breached by buffalo in the past, cattle in the buffer zone are vaccinated biannually with an inactivated trivalent vaccine. Historically, the FMD vaccine contained five virus strains (two SAT-1, two SAT-2 and one SAT-3) to ensure adequate antigenic coverage and protection against the highly variable field strains in circulation in buffalo. As effective control through vaccination requires the continuous development of vaccines that better match field and outbreak strains, it is important to identify suitable vaccine strain candidates for rapid formulation of novel vaccines in response to outbreaks and/or the detection of new virus variants. Vaccine strain selection is, however, time-intensive as only a small number of viruses meet the stringent requirements of rapid adaptation to monolayer and suspension cell cultures, sustained high virus yield and genome stability over successive passages. In this study, four SAT-type viruses that are not part of the current FMD vaccine formulation for South Africa were assessed for their vaccine strain potential. PCR of the P1 region using NCR1 and WDA primers and Sanger sequencing was done as an initial verification step. Each low-passage virus was then adapted to baby hamster kidney (BHK) 21 monolayer cells, after which the virus was passaged three times on BHK-21 suspension cells. Samples from the infected suspension cell cultures were collected at regular intervals from 16 to 40 hours post-infection to monitor changes in the tissue culture infectious dose $(TCID)_{50}$. Lastly, next-generation sequencing was employed to determine the genome stability of each virus. Integration of the resulting data on changes in variant composition associated with adaptation to suspension cell culture, and virus yield allowed for ranking of the candidate vaccine strains assessed in this study. The approach and results will ultimately guide the selection of the virus isolates that are best-suited to vaccine production in BHK-21 suspension cell culture.

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List of abbreviations

AEC	Animal ethics committee
ARC-OVR	Agricultural research council- Onderstepoort veterinary research
BEI	Binary ethyleneimine
BHK	Baby hamster kidney
BSL3	Biosafety level 3
cDNA	Complimentary deoxynucleic acid
CPE	Cytopathic effect
DALRRD	Department of Agriculture, Land Reform and Rural Development
DNA	Deoxynucleic acid
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus
GAG	Glycosaminoglycan
HPI	Hours post-infection
HS	Heparan sulfate
IB-RS	Instituto Biologico Rim Suino
KNP	Kruger National Park
MCS	Master Cell stock
MOI	Multiplicity of infection
PCR	Polymerase chain reaction
PD50	Protective doses 50
REC	Research ethics committee
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-Polymerase chain reaction
SAT	South African Territories
SOP	Standard operating procedure
TADP	Transboundary animal diseases programme
$TCID_{50}$	Tissue culture infectious dose 50

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1. GENERAL INTRODUCTION

Foot-and-mouth disease (FMD) is an infectious viral disease, affecting both cloven hoofed livestock (cattle, sheep, goats and pigs) and wildlife (African buffalo, impala and kudu antelope, amongst others) (Dion et al., 2011). It is caused by the foot-and-mouth disease virus (FMDV) and it is transmissible through the air or by direct contact (Schook et al., 2014). High fever, lameness and vesicular lesions to hooves, tongue and teats are some of the symptoms associated with the disease (Nfon et al., 2017). The mortality of this disease differs between species and age groups, with low mortality in adult animals and high mortality in young ones, depending on the infecting serotype and virus strain (Abubakar et al., 2013).

FMD remains an important disease of livestock worldwide because of its adverse effect on animal production and animal trade. It results in decreased milk production and causes weight loss and loss of productivity in draft animals (Grubman and Baxt, 2004). Because of this, FMD-endemic countries face trade restrictions from export partners resulting in substantial economic losses (Knight-Jones and Rushton, 2013).

FMD is endemic to South Africa because African buffaloes (*Syncerus caffer*) in the Kruger National Park (KNP) are persistently infected with the three South African Territories (SAT) serotypes and studies have shown that the buffaloes play a role in outbreaks outside the KNP (Vosloo et al., 2002c). Historically, most outbreaks in the areas adjacent to KNP were caused by SAT-2, followed by SAT-1, and less so by SAT-3 (Vosloo et al., 2006). A large number of buffalo become infected with all three SAT serotypes at a young age and they may continue to be carriers throughout their lifespan (Vosloo et al., 2002c). Studies have shown that SAT-1 rather than SAT-2 or SAT-3 is recovered more often in buffaloes and 60% of them go on to be carriers and may maintain the virus up to 24 years (Condy et al., 1985). In order to constrain the virus and permit trade, South Africa is divided into zones of presence and absence of FMD to protect its FMD-free status and allow for trade. Control measures to contain FMD to the infected zones include the restriction of animal movement, fencing to separate buffalo and cattle and vaccination with a trivalent vaccine, and inspection of livestock at varying time intervals (Bruckner et al., 2002b, Vosloo et al., 2002b).

Vaccination as a form of control and eradication of FMD has some success in different parts of the world (Europe, North America and some Asian countries) (de los Santos et al., 2018). Although effective protection through vaccination is hard to achieve, owing to the antigenic diversity of the virus, it remains an important part of control strategies, especially in regions where the implementation of a stamping-out policy is not an option.

Commercially available vaccines are mostly inactivated FMDV produced by cultivating the live virus in cell cultures. Wild type field strains are adapted to cell-cultures through various passages and the adapted strains become possible vaccine candidates (de los Santos et al., 2018).

The adaptation of an FMDV strain (potential vaccine candidate strain) in cell cultures involves multiple passage of the virus on cells leading to mutations associated with adaptation. These changes may be due to the use of different cell receptors upon adaptation. When these changes accumulate in important antigenic sites, they could affect the antigenicity of the virus during vaccine production. The aims and objectives of this research was to adapt four SAT 1 serotype strains of FMDV in baby hamster kidney (BHK) suspension cells and monitor genomic stability and genetic mutations associated with successive passage and adaptation.

2. LITERATURE REVIEW

2.1. Introduction

FMD is an infectious viral disease, affecting both cloven hoofed livestock (cattle, sheep, goats and pigs) and wildlife (primarily African buffalo, and impala antelope) (Dion et al., 2011). It is caused by a small, single-stranded positive-sense RNA virus, FMDV, classified within the *Aphthovirus* genus, family *Picornaviridae* (Schook et al., 2014). This highly-infectious virus, which is transmissible through the air or by direct contact causes high fever, lameness and vesicular lesions on the hooves, tongue and teats of affected animals; symptoms that assist with identification of the disease (Nfon et al., 2017). Morbidity and mortality vary greatly between affected species and age groups, and infecting virus. However, mortality in adult animals is generally low, with higher levels of mortality only occurring in younger animals (Abubakar et al., 2013).

2.2. Epidemiology

2.2.1. Distribution of FMD

FMDV exists as seven distinct serotypes; the three SAT serotypes 1, 2 and 3, the three Eurasian serotypes A, O, C, and Asia-1 (Carrillo et al., 2005). Of these, one is extinct in the field (serotype C) and five are found on the African continent (SAT 1, SAT 2, SAT 3, O, and A). In contrast, only three of the seven serotypes occur in Asia, namely A, O and Asia-1. The most widely distributed serotypes, globally, are A and O, and the least common, and now considered extinct is serotype C (Casey et al., 2014). SAT 1, 2 and 3 are endemic to most African countries south of the Sahara (Bastos et al., 2003) with all southern African FMD outbreaks have been due to these three serotypes, with few exceptions (Vosloo et al., 2002b).

2.2.2. FMDV topotypes

The intrinsically high mutation rate of the virus combined with long-term persistence in animals, especially in African buffalo (*Syncerus caffer*) has caused the emergence of virus subtypes that are regionally distinct. This regional genetic variation is known as the viral topotype (Samuel and Knowles, 2001). The topotype classification sub-divides the FMD serotypes based on geographic locality (Bastos et al., 2003, Vosloo et al., 2005). According to Vosloo and colleagues (2002a), SAT 2 viruses have the highest number of topotypes followed by both SAT 1 and O serotypes. Serotype C has the lowest number of topotypes recorded to date (Knowles and Samuel, 2003). In

southern Africa, the selection of FMD vaccine strains is based on viruses circulating in local African buffalo populations. For many countries in the region the existence of multiple serotypes and topotypes (within each serotype), poses an immense challenge, therefore the selection of vaccine candidate strains that address the associated antigenic diversity is important.

2.2.3. Wildlife's impact on FMD in Southern Africa

Wildlife plays a role in the epidemiology of FMD in different parts of Africa (Thomson et al. 2003). The existence of large numbers of wildlife and the close contact at the interface between wildlife and livestock has resulted in a suitable environment for the transmission of FMDV (Casey et al., 2014). The presence of persistently infected buffalo populations and the long association between buffalo and FMDV are likely reasons for the high levels of FMD virus diversity in Africa. A clear example of this can be found in South Africa in the buffalo population of the Kruger National Park (KNP). FMD is endemic in the KNP, a wildlife conservation area that has an African buffalo (*Syncerus caffer*) population of ~48 000 animals that are known maintenance hosts of the SAT serotypes and which serve as reservoirs of infection (Dion et al., 2011). Maintenance of SAT serotypes in buffaloes of between 5 to 24 years has been demonstrated and most calves are infected with all three SAT-types prior to reaching one year of age (Vosloo et al., 2002). Although control measures to prevent close contact between wildlife (especially buffaloes) and livestock exist, buffaloes have on several occasions escaped the park and caused outbreaks in livestock, although studies have shown that this pathway is a rare event (Dion et al., 2011)(Thomson, 2003). Currently, less is known about other wildlife species such as kudu and impala antelope, and other cloven-hoofed species in the epidemiology of FMD, but their role as intermediary hosts, rather than maintenance hosts has been highlighted in a number of studies (Casey et al., 2014).

2.2.4. Control of FMD in South Africa

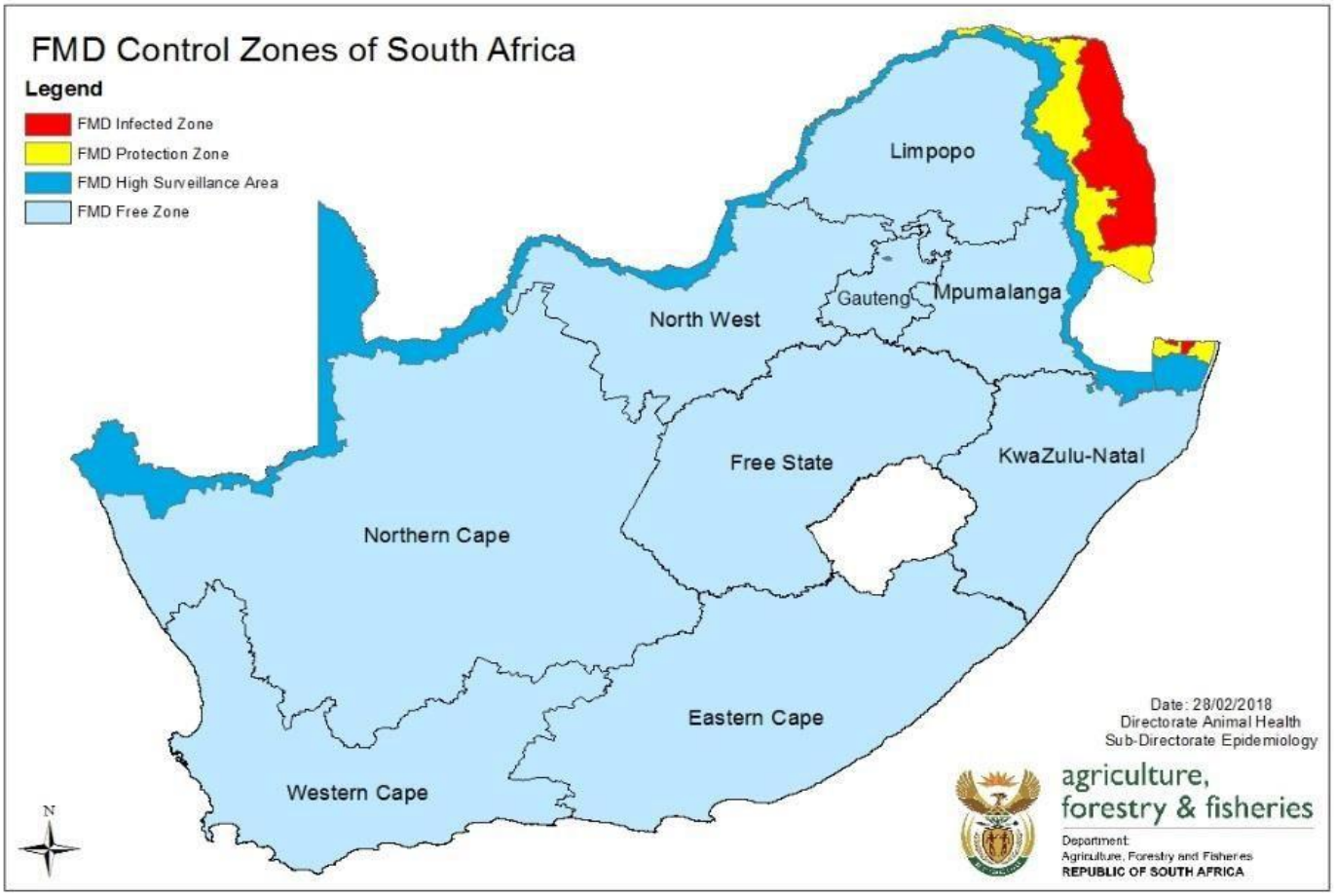

Figure 2.1 A map of FMD control zones showing the “infected zone”, “Protection zone, and FMD high surveillance area (DAFF, 2019).

According to the Animal Disease Act of South Africa (35/1984), FMD is considered a controlled disease for which there is a stringent FMD control policy. South Africa is divided into zones of FMD absence and presence (Bruckner et al., 2002b). KNP which is positioned the north-east of South Africa, is an iconic 2-million-hectare wildlife conservation site that is endemic for the disease. It is, therefore, considered an “infected zone” and the areas adjacent to the western and southern borders of KNP, stretching from Mpumalanga Province in the south to the Limpopo Province in the north are classified as the protection zone of approximately 480 km long and 10-20 km wide. The protection zone is further divided into protection zone with vaccination

and protection zone without vaccination. FMD high Surveillance areas are adjacent to these control zones (Sirdar et al., 2021, Bruckner et al., 2002b, Vosloo et al., 2002a).

The infected zone is endemic for FMD because of the presence of infected African buffalo which as maintenance host for the three SAT serotypes, represent a permanent source of FMD virus. Separation of wildlife and livestock in areas adjacent to the infected zone, is key to limiting outbreaks and relies on maintenance of single fences that were erected around the KNP to prevent such contact. In some cases, the use of fences is not enough. The escape of African buffaloes from the park due to fence damage has occurred on multiple occasions (Vosloo et al., 2002c). Each breach compromises the efficiency of the fence control strategy, which represents an important first line of defence. For this reason, a protection zone and FMD high surveillance area, respectively indicated in yellow and dark blue in Figure 2.1, exist adjacent to the KNP and collectively they make up the FMD control zones. Cattle in the protection zone with vaccination are vaccinated every four months with a trivalent FMD vaccine comprising of serotypes SAT 1, 2 and 3 (Sirdar et al., 2021). Vaccinated cattle are branded and they, together with cattle meat products are prohibited from movement to the FMD free zone. In addition, animal health technicians and state veterinarians inspect livestock at varying time intervals, primarily at state-run dip tanks. Livestock cannot be moved out of the surveillance zone unless tested and declared FMD-negative prior to movement to other places within the country (Bruckner et al., 2002a, Vosloo et al., 2002a).

2.2.5. Clinical signs and symptoms in domestic animals

FMD affects diverse cloven-hoofed animals resulting to different clinical signs depending on species, age and breed of the animal and on the infecting FMDV strain. In infected cattle, the first sign is a fever of 40°C and higher, followed by the appearance of vesicles and viral lesions in the tongue and mouth epithelia. The affected mouth areas include muzzle, gums and dental pad, resulting in drooling from the mouth and eating avoidance. Lesions also occur on the hooves. In pigs the most common lesions are seen on the hooves, but some are seen on the snout and other areas, although these are not common. Mouth lesions are also uncommon in sheep and goats. In most susceptible domestic animals, abortions can occur in pregnant animals (Nfon et al., 2017).

2.3. Aetiology

2.3.1. Taxonomy and Classification

FMDV, the causative agent of foot-and-mouth disease is a member of the family *Picornaviridae* within the genus *Aphthovirus*. The *Picornaviridae* family consists of viruses with small positive-sense RNA genomes that infect insects, plants and animals. The FMDV is distinguished as an *Aphthovirus* by its physicochemical properties such as sensitivity to acid inactivation (pH less than 6.8), high buoyant density and presence of a poly(C) tract in the 5' untranslated region (UTR) of the RNA genome, to name a few (Rowlands, 2020).

2.3.2. FMDV genome organization

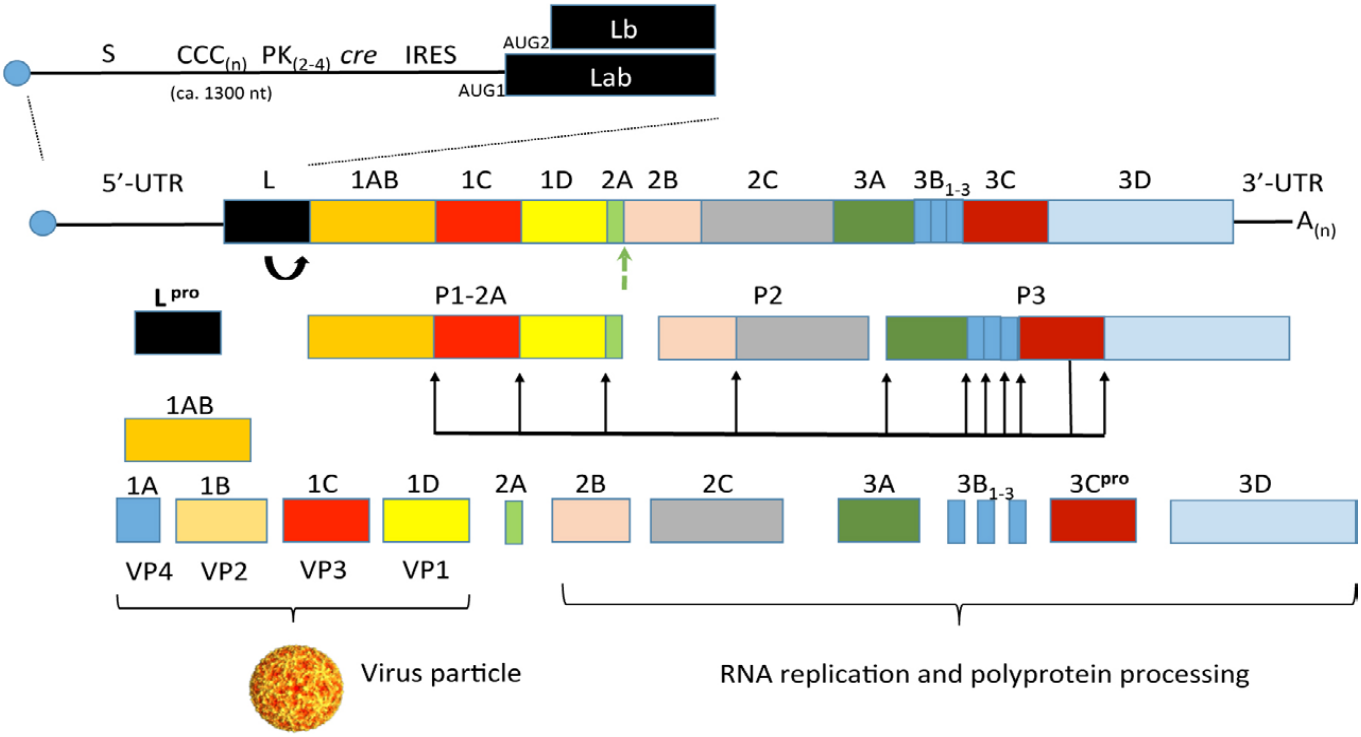

Figure 2.2 FMDV genome organisation summarising the most important features of the FMDV RNA genome and encoded proteins (Martinez-Salas and Belsham, 2017).

The FMDV particle has a protein shell (capsid) made up of 60 copies of structural proteins, namely VP1, VP2 VP3 and VP4 which are all encoded within the P1 region of the genome (Figure 2.2). The length of the genome is approximately 8.5 kb and is made up of a large (7000 nucleotides) open reading frame (ORF) encoding both the structural and non-structural proteins, 3' untranslated region (3'UTR) with a poly (A)

tail, a 1300 nt 5' untranslated region (5'UTR) and a virus-encoded peptide called VPg (3B) attached to the terminal nucleotide at the 5 prime-end (see figure 2.2). The genomic RNA, while protected by the virus particle when outside the host cell, enters the cell and is replicated in the cytoplasm with high efficiency by a virus RNA-dependant RNA polymerase (3Dpol) (see Figure 2.2) (Martinez-Salas and Belsham, 2017).

2.3.3. The structure of the virion

General structure of the FMDV virion

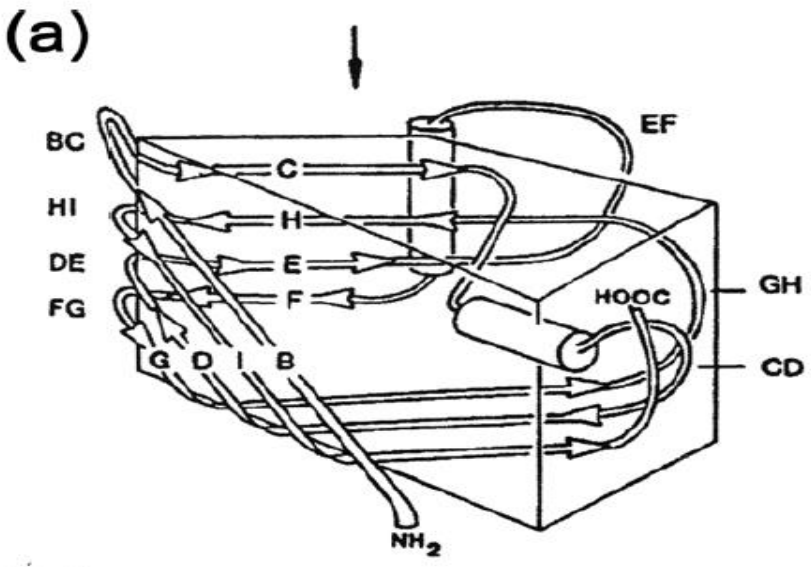

Figure 2.3 (a) Representation of the VP1, VP2 and VP3 fold of FMDV with single letters and letter pair representing β-strands and loops respectively (Mateu, 2017).

A combination of x-ray crystallography, cryo-electron microscopy and nuclear magnetic resonance spectroscopy methods have been used to determine the structure of the FMDV virion (Mateu, 2017). The virion is a spherical hollow protein capsid, 30 nm in diameter, carrying the genomic RNA and is icosahedral in symmetry. The virion capsid is made up of 60 copies of structural proteins VP1, VP2, VP3 and a polypeptide VP4 (Mateu, 2017). Structural proteins VP1, VP2, and VP3 are similar in size and shape and their polypeptide chain (between 210 - 220 residues in length) folds into the same eight-stranded β-barrel form (Figure 2.3). The eight β-strands then

fold to form a wedge of two β-sheets containing four β-strands (C, H, E, F and B, I, D, G). This wedge is the flat side of the trapezoidal VP and the inner capsid surface containing the N-termini. The β-strands are connected by loops of different lengths, named by the two strands, they connect. The BC, HI, DE and FG loops are short and found at the narrow side of the wedge whereas GH, EF and CD are longer and found on the outer surface of the capsid (Figure 2.3; (Mateu, 2017).

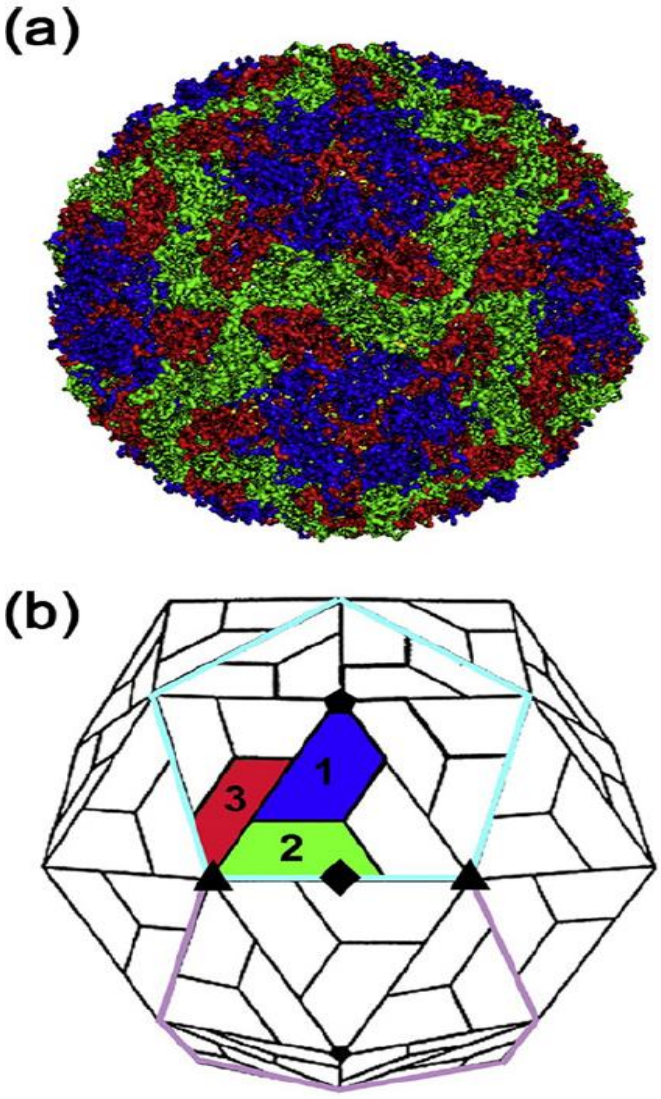

Figure 2.4 (a) the structure of the FMD virion with VP1, VP2 and VP3 respectively coloured blue, green and red. (b) FMDV capsid representation, showing its icosahedral organisation, VP1, VP2 AND VP3 numbered 1, 2, and 3 belonging to the same protomer. Cyan and violet lines show two pentamers (Mateu, 2017)**.**

Structural proteins VP1, VP2 and VP3 fit together in a trapezoidal held together by non-covalent interactions to form a substructure called a biological protomer (with VP4

polypeptide running along the inner surface). Five biological protomers fit together at each capsid's five-fold axis through non-covalent interactions to form a pentamer. The pentamer fits to adjacent pentamers non-covalently through protein-protein interfaces to form the complete capsid of the virion (Figure 2.4b, (Fry et al., 2005).

The highly important GH loop of VP1

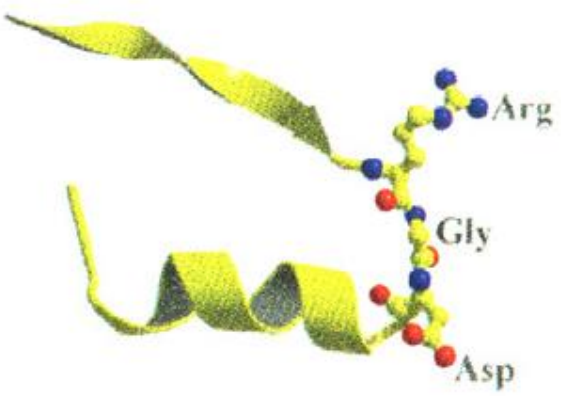

Figure 2.5 The structure of a G-H loop of VP1 of a chemically reduced serotype O FMD virus showing the short β-strand region, followed by the highly conserved Arg-Gly-Asp (RGD) motif, followed by a short 3_{10}-helix region (Mateu, 2017).

The GH loop of VP1 structural proteins is a very important immunogenic region as it contains major antigenic regions and binding sites for integrin receptors (briefly reviewed below) of the host cells. The crystallographic structure of the VP1 G-H loop in most studied FMD viruses are disordered therefore invisible and the only structure that could be determined was that of a serotype O virus that had been chemically reduced, thus breaking the VP1-VP2 disulfide bond (Logan et al., 1993). The G-H loop is made up of a short β-strand stretch of residues, followed by a central Arg-Gly-Asp (RGD); a highly conserved motif that functions in integrin binding, and has an open conformation, followed by a short 3_{10} -helix region (Figure 2.5, (Burman et al., 2006, Logan et al., 1993).

2.3.4. FMDV receptors: Cell recognition

The first step to a successful infection of a host animal with FMDV is the attachment of the virus particle to the host cell. This happens through the attachment of FMDV capsid proteins to one of multiple host cell receptor molecules on the plasma

membrane. This is different to cell culture where a limited number of receptors are utilized by the FMDV. Numerous studies done by different researchers revealed that all seven serotypes use either a primary receptor or an alternative secondary receptor and some variants can even use a third alternative receptor (Lawrence and Rieder, 2017).

2.3.4.1 Integrins as the primary FMDV receptor

Integrins are transmembrane proteins that are made up of two subunits, an α subunit and β subunit. Eighteen α subunits and eight β subunits are known and these generate 24 heterodimers when combined in different combinations. Eight out of the 24 integrins utilize the RGD motif as a recognition sequence (LaPointe et al., 2014). A study investigating the binding of FMDV virion to cell culture, showed that virus virions partially digested with trypsin resulted in them being unable to attach to cells in cell cultures, rendering them non-infectious (Barteling et al., 1979). On close analysis, it was revealed that a single cut at the cleavage site Arg 144 on the G-H loop of VP1 (part of RGD tripeptide) resulted in non-attachment of the virion to the cells in culture (Robertson et al., 1983). In a number of successive studies, Pierschbacher and colleagues showed that the Arg-Gly-Asp (RGD) tripeptide sequence on the G-H loop might be a recognition sequence for integrins (Pierschbacher and Ruoslahti, 1984a, Pierschbacher and Ruoslahti, 1984b, Pierschbacher et al., 1985). In the years between 1994 and 1997 reverse genetics methods gave the most reliable insights through mutations or deletions in the RGD sequence of infectious cDNA clones which resulted in the production of non-infectious virions that cannot bind to cells in culture and cannot cause disease in susceptible animals (Mason et al., 1994, Rieder et al., 1996, Leippert et al., 1997).

2.3.4.2 Heparan Sulfate as an alternative/secondary FMDV receptor

Heparan sulfate (HS) is a glycosaminoglycan (GAG) that is made up of disaccharides of hexuronic acid and D-glucosamine repeats forming a linear polysaccharide molecule, expressed in huge quantities on the cell surface (Simon Davis and Parish, 2013). Jackson and colleagues (1996) showed that FMDV serotype O could not efficiently infect cells in culture when HS inhibiting agents are present, even in the presence of αvβ3 integrin (Jackson et al., 1996). Another study to support this was done by Neff and colleagues in 1998, where they demonstrated that FMDV adapted

to cell cultures could utilize the HS receptor to infect cells (Neff et al., 1998). Most studies done on the utilization of HS as an alternative receptor, all demonstrated amino acid substitutions to the structural proteins of the capsid as the critical driving force to the adoption of HS receptor binding and this happens during cell culture adaptations of the virus (Lawrence and Rieder, 2017).

2.4. Genetic variation in FMDV

2.4.1. Introduction

The selection of FMD vaccine candidate strain is complicated by the genetic and antigenic variation caused by RNA viruses in host cells. This is shown by numerous studies done by Domingo and colleagues, which reinforced the quasispecies concept. The concept suggests that RNA viruses including FMDV, use genetic variation mechanisms (high mutation rates, high yields and short replication time) to form a population of multiple variants (Domingo et al., 1985, Domingo and Holland, 1997). FMDV field isolates in many cases are made up of a population of genetically and antigenically different variants. In addition, the serial passage of FMD virus strain in BHK cells during adaptation results in host-cell selection of antigenic variants that are slightly different from the wild type virus (Bolwell et al., 1989, Sobrino et al., 2001) further complicating the selection of vaccine candidate strains.

2.4.2. The basics of quasispecies concept

All simple RNA and some DNA viruses share a common feature amongst them and that feature is error-prone replication. This is largely caused by absence or non-efficient proof-reading and post-replication repairs during replication, resulting in varying genetic composition of the virus population (a swarm of virus variants). These virus variants can have different genetic compositions (because of mutation) without changing the consensus sequence of the virus population. The virus variants in the population are known as viral quasispecies defined as a group of related variants with non-similar genomes (genetic variation). This is the basic concept (mutations as a necessity during replication) of the quasispecies theory phrased by Eigen and Schuster (Domingo et al., 2016).

2.4.3. Mechanisms for genetic variation

Viruses like FMDV make use of the variation mechanisms used by other life forms, which includes mutation and recombination. However, in contrast to eukaryotic life forms, the amount of change and the time taken for the change to be observed is markedly different. To give an example of this in viruses, Drake and Holland (Drake and Holland, 1999) reported that most RNA viruses mutate at 10^{-3} to 10^{-5} substitutions for each nucleotide copied, which is up to a million-fold higher than the mutation rate in cellular DNA during normal replication. Another source of variation is RNA recombination, as demonstrated for FMD virus genomes by King and colleagues (1982).

2.4.4. Quasispecies in FMDV

Evidence of genome variation in FMDV was seen in earlier studies by Dietzschold (with colleagues) and Robson (with colleagues) when analysing viral RNA. Ribonucleic acid from FMDV serotypes A, O and C had up to 70% nucleotide sequence identities and more than 70% nucleotide sequence identity in RNA of different FMDV subtypes in serotypes A and O (Dietzschold et al., 1971, Robson et al., 1977). Frisby and colleagues also found nucleotide sequence changes in FMDV RNA within the same serotypes (Frisby et al., 1976). A study done by Sobrino and colleagues showed multiple genetic variant formation of FMDV during the passage of a virus isolate with one parental RNA sequence. The study highlighted three observations which are (a) the production of a range of related variants of a clone when passaged in two cell lines, (b) dominance of certain genomic sequences on newly generated variants, (c) adaptation to cell cultures concomitant with an increase in viral load (Sobrino et al., 1983). All of these studies gave evidence of genetic variation in FMDV. This poses a major challenge to control FMDV outbreaks by vaccination because variants are generated during replication that are capable of vaccine-induced immune response escape; a selective advantage that results in dominance.

2.5. FMD vaccine development

2.5.1. Vaccine production

Commercially available vaccines are mostly inactivated virus vaccines produced by cultivating live FMDV in tissue cultures such as baby hamster kidney (BHK) 21 cells whether stationery or in suspension, in large quantities. The virus is then harvested,

inactivated by agents such as binary ethyleneimine (BEI) and concentrated by precipitation or filtration. Vaccine formulations include one or more virus serotypes plus a buffer and an adjuvant (oil or aluminium hydroxide). The produced vaccine must have a minimum potency of three protective doses. PD_{50} the most used potency test for FMD vaccine potency, is calculated as a recommended vaccine dose that protects 50% of cattle challenged by inoculating with live virus that is similar to the vaccine strain, 21 days after vaccination (de los Santos et al., 2018, Kitching et al., 2007). Although inactivated virus vaccines produce a limited immune response, they are still the preferred method for FMD control through vaccination. Booster vaccination and annual re-vaccinations are recommended (de los Santos et al., 2018) although some studies have suggested that even a 4 - 6 month interval between vaccinations is not sufficient to prevent an outbreak (Woolhouse et al. 1996).

2.5.2. Adaptation of FMDV to BHK cell cultures

The principle of virus adaptation involves passaging a virus multiple times in an established cell line until the virus reaches high titres (Dill and Eschbaumer, 2020). FMDV is no different, and the preferred cell line is the BHK cell line. Mowat and Chapman (1962) showed that an efficient method to grow and titrate FMDV was in BHK 21 clone 13 developed by MacPherson and Stoker (Doel, 2003). Although laborious, it is used by many in the production of FMDV vaccines. Large scale production of FMDV can also be adapted to grow on BHK suspension cells in large-scale fermenters to eliminate the “multiple open operations” involved with the monolayer cell line (Doel, 2003).

During the adaptation process in cell cultures, FMDV acquires mutations that allow the virus to not only use integrins as receptors (primary receptors) but HS as secondary receptors. This is a required process in vaccine production as it promotes adaptation (Dill and Eschbaumer, 2020). On the other hand, it is ideal to adapt the virus within the fewest passage numbers possible to ensure that sequence variation in the seed virus remains low (Anil et al., 2012) thereby avoiding/limiting changes in the amino acid sequence of viral proteins (Dill and Eschbaumer, 2020).

2.5.3. Viral titre determination assay

During the process of virus adaptation, determination of the strength of infectious virus (infectivity) to test the level of adaptation is important. Virus titre is one way to

determine the strength of infectious virus and the tissue culture infectious dose 50 ($TCID_{50}$) assay is a common assay to determine viral titre. $TCID_{50}$ is a form of an end-point dilution assay. It determines the ratio between infected and un-infected cells in a number of plate wells when host cell cultures are infected with the virus of interest at various virus dilutions. The plate is incubated for a pre-determined period, observed under a microscope for the presence/absence of cytopathic effects (CPE) and the point at which 50% of the cells are infected is determined using the Reed and Muench method (Smither et al., 2013); the titre is thus calculated as $TCID_{50}$ per millilitre of virus sample ($TCID_{50}$/ml). The level of adaptation of the virus is investigated in different samples generated during the adaptation process and is related to the titre of the virus, the higher the titre, the higher the adaptation level.

2.6. Problem statement

In South Africa, one of the strategies to control FMD is through vaccination with a vaccine containing the three SAT serotypes. The Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR) Transboundary Animal Diseases Programme (TADP) is tasked with developing FMD vaccine for the country. This study is part of a bigger FMD vaccine production project that aims to produce suitable FMD vaccine for South Africa.

Literature suggests that FMD SAT 1 and 2 serotypes are the most common serotypes associated with FMD outbreaks in South Africa. Long-term persistence of FMDV in wildlife has caused genetic and antigenic variation, and adaptation to BHK cells in a laboratory setting results to selection of variants that may differ from those of the wild type thus complicating the selection of a candidate vaccine strain. The focus of the study was to select an SAT 1 serotype strain that is not included in the current FMD vaccine (ARC-OVR) by assessing four candidate vaccine strains for adaptation to large scale production in BHK suspension cell cultures. The potential benefit is that an additional SAT 1 FMD vaccine candidate similar to the southern African region topotypes will be available for possible inclusion in the trivalent vaccine.

2.7. Aim and objectives

2.7.1. Aim

The aim of this study is to select and adapt four SAT 1 strains of FMDV to BHK suspension culture and assess them for vaccine strain potential. This will be achieved by:

- Selecting four SAT 1 virus strains that are currently not included in the South African FMD vaccine but which match field strains in circulation in South Africa.
- Adapting the selected virus strains to BHK adhesive/monolayer cells, followed by three successive passages on BHK suspension cells.
- Using next generation sequencing of the entire genome of the adapted virus strain/s to identify genetic mutations associated with adaptation to the BHK suspension cells.
- Integrating data on virus genome variation/stability and the speed of adaptation to rank the vaccine strain candidates.

2.8. Ethical and biosafety consideration

Research and animal ethics approval for the project was obtained from the University of Pretoria's Research Ethics Committee and Animal Ethics committee (REC 132-20). In addition, Animal ethics approval was obtained from Agricultural Research Council's Animal Ethics Committee (AEC 19.14). A section 20 application was also obtained from Department of Agriculture, Land Reform and Rural Development (DALRRD).

All virus work was conducted at Agricultural Research Council - Onderstepoort Veterinary Research (ARC-OVR) biosafety level 3 (BSL3) facility at Transboundary Animal Diseases Program, under strict standard operation procedure protocols.

3. MATERIALS AND METHODS

3.1. Virus samples

Four different low-passage FMDV SAT 1 samples were obtained from the National FMD Virus Bank at ARC-OVR. Each 1 ml vial of FMDV SAT 1, grown on Instituto Biologico Rim Suino (IB-RS)-2 cells (of porcine kidney origin) was given an in-house identification, viz. SAT1V1, SAT1V2, SAT1V3 and SAT1V4. Virus samples were stored at -80°C until use.

3.2. Cell cultures

3.2.1. Complete cell seeding medium

Complete cell seeding medium was prepared by using Glasgow's Minimal Essential Medium (Sigma, product number: G6148) supplemented with 10% bovine serum (Cell Sera, cat: 102131610), 10% tryptose phosphate broth (Bacto, cat: 260200) and 3% lactalbumin hydrolysate (Bacto, cat: 259961).

3.2.2. Complete virus seeding medium

Complete virus seeding medium was prepared by using Glasgow's Minimal Essential Medium (Sigma, product number: G6148) supplemented with 1% bovine serum (Cell Sera, cat: 102131610), 10% tryptose phosphate broth (Bacto, cat: 260200) and 3% lactalbumin hydrolysate (Bacto, cat: 259961).

3.2.3. BHK cell cultures (adherent)

BHK 21 cells which are adherent cells, were obtained from the ARC-OVR tissue culture laboratory as 1 ml vials of master cell stock passage number 2 (MSC+2). The cells were seeded and cultivated in 75 cm^2 and 175 cm^2 flasks (CORNING®Flask, product number: 430720/431079), with complete cell seeding medium and incubated at 37°C until they reached confluency of 80 – 90 %. Sterility tests were done by inoculation of broths (nutrient broth, thioglycolate broth and tryptic soy broth, Merck) with 1 ml of cell samples taken from each flask at the end of the incubation time. These cells were used for virus adaptation, virus titration and to maintain cell seeding stocks.

3.2.4. BHK cell cultures (suspension)

BHK 21 cells in suspension were obtained from ARC-OVR's tissue culture laboratory as 1 ml vials of master cell stock passage number 1 (MCS+1). The cells were seeded and cultivated in 1750 cm^2 Corning roller bottles (CORNING®, product number: 430699) to a maximum cell suspension of 1500 ml, with complete cell seeding medium

and incubated at 37°C until they reached a minimum cell count of 2.5 million cells per millilitre. Sterility tests were done by inoculation of broths (nutrient broth, thioglycolate broth and tryptic soy broth, Merck) with 1 ml of cell samples taken from each flask at the end of the incubation time. These cells were used for virus adaptation and to maintain cell seeding stocks.

3.3. PCR and Sanger sequencing

Viral RNA was extracted using the QIAmp Viral RNA mini kit (QIAGEN) following the manufacturer's instructions from the four low-passage FMDV SAT 1 viruses, at the start of the experiment. Complementary DNA (cDNA) was synthesised using the One *Taq*® One-step RT-PCR kit (BioLabs inc) following the manufacturer's instructions, targeting the P1 region of the FMDV, using NCR1 forward (5'TACCAAGCGACACTCGGGATCT3') and WDA reverse (5'GAAGGGCCCAGGGTTGGACTC3') primers at an annealing temperature (Ta) of 55°C. The PCR products were run on a 1.5% agarose gel to confirm amplification of the P1 region of about 2300 bp. The amplicon of the expected size was purified from the agarose gel using the QIAquick® Gel extraction kit (QIAGEN). The recovered P1 region of all virus samples was inactivated at 60°C for 30 minutes, in accordance with relevant protocols for removal of samples from the BSL3 laboratory, and submitted to Inqaba Biotech for Sanger sequencing.

3.4. Virus adaptation studies

3.4.1. First passage on BHK adherent cells

Five 75 cm^2 flasks with BHK adherent cells (90% confluent) at the end of their incubation time were taken and observed under the microscope to check their quality and sterility. Spent media were removed from the flasks and 20 ml of fresh virus seeding media was added. The pH was adjusted to 7.40 and 200 µl of each virus (SAT1V1, SAT1V2, SAT1V3 and SAT1V4) was then added to each flask; the fifth flask was used as a negative control. All flasks were labelled and incubated at 37°C and inspected at 15-hour intervals until CPE was observed.

Once 90% CPE was observed and cells were starting to detach, the contents of each flask were transferred to 50 ml centrifuge bottles (CORNING®, Product number: 430921). The flasks were frozen at -80°C for two hours then returned to room temperature. Each flask was shaken vigorously to detach all cells. The contents were

again transferred to 50 ml centrifuge bottles and centrifuged to pellet cell debris. The clarified supernatant was dispensed into 1 ml cryogenic tubes (Thermo Scientific, Cat number: 5000-1020) and stored at -80°C. These stocks correspond to the first passage on BHK adherent cells.

3.4.2. Viral titre determination

Virus samples from the first passage were titrated according to the ARC-OVR standard operating procedure (SOP: QC002 Titration test for the FMDV samples in BHK21C13 cell cultures) and the viral titres were determined and recorded.

3.4.3. Adaptations on BHK adherent cells

The first passage was followed by serial passages on BHK adherent cells until each virus reached a titre of 7 $TCID_{50}$/ml at a multiplicity of infection (MOI) of 1:10. The passages were done the same way as the initial passage with the only difference being the use of virus titres (determined after the first BHK-21 passage) to calculate the virus volume needed to infect cells for the next passage. Viral titres were determined after each passage, as previously described.

3.4.4. Adaptations on BHK suspension cells

Passage in adherent cells was followed by three passages in BHK suspension cells for all four virus samples. Suspension cells for the first passage were prepared by taking one 1750 cm^2 Corning roller bottle with BHK suspension culture (1500 ml cell suspension) and allowing the culture to settle at 8°C overnight, after which the spent medium was decanted and replaced with virus seeding medium to form a 1500 ml cell suspension. Four 200 ml cell suspension aliquots were each transferred to 850 cm^2 Corning roller bottles (CORNING®, Product number: 430849). Each of the four roller bottles were infected with one of the four virus samples at a multiplicity of infection (MOI) of 1:10. The final viral titre (from the adaptations on adhesive cells) was used to determine the amount of virus for the infection. The cultures were incubated with rotation (30 rpm) at 37°C for 14 hours. Samples were taken at 14 hours post-infection (hpi) and at 2-hour intervals until 24-hours post infection. These samples were observed for CPE and cell counts were done according to ARC-OVR standard operating procedures (SOP: VP006 Cultivation of SAT type viruses in BHK38 Clone E3 in suspension cultures, VP004 Cell counting and determination of cells viability using trypan blue exclusion method). Depending on the amount of CPE and cell

counts, cultures were left to incubate until 37-40 hours post-infection. Left over cultures were aliquoted in sterile Nalgene media bottles (Merck, Product number: Z364533) bottles and stored at -80°C.

The samples generated were titrated according to the ARC-OVR standard operating procedure (SOP: QC002 Titration test for the FMDV samples in BHK21C13 cell cultures). The viral titres were determined and recorded.

The second and the third passage on suspension cells was carried out in the same way as the first passage on suspension cells.

3.5. Next Generation Sequencing of viral RNA

Three samples of each of the four SAT1 virus were selected for analysis through Next Generation Sequencing (NGS). This included the original virus samples, first passage on suspension cells and third passage on suspension cells, thus 12 virus samples in total.Total RNA was extracted from the cell culture virus passages using the QIAmp Viral RNA mini kit according to manufacturer's instructions. RNA was eluted in 140 µl of nuclease-free water and quantified using the Qubit RNA High Sensitivity (HS) Assay Kit (Life Technologies). Contaminating genomic DNA (gDNA) originating from the cell culture was depleted from extracted viral RNA samples through the addition of rDNase1 using the DNA-free DNAse kit (Life Technologies). Briefly, 5 µl of DNase Buffer and 1 µl of rRDNase1 (2 U) was added to 10 µg of viral RNA, and incubated at 37°C for 30 minutes. The enzymatic reaction was terminated by the addition of inactivation agent as per the manufacturer's protocol and the sample was incubated for a further two min at room temperature.

First-strand cDNA synthesis was performed using Superscript III First-Strand Synthesis System (Life Technologies) according to the manufacturer's protocol. Briefly, 10 µl of DNase-treated total RNA was combined with 50 ng/µl random hexamers (Life Technologies), 10mmol dNTPs (Life Technologies) and nuclease-free water. Reactions were incubated at 65°C for 5 min. Two hundred units of SuperScript III enzyme (Life Technologies), 40 U RNaseOUT (Life Technologies), 0.1 M dTT (Life Technologies) and 25 mM MgCl2 were added and the reaction was incubated at 50°C for 50 min, followed by incubation with 2U RNase H (Life Technologies) at 37°C for 20 min. Second-strand synthesis was performed using NEB Second Strand Synthesis kit (NEB) as per manufacturer's instructions using 20 µl of cDNA.

The dsDNA was purified using Illustra GFX DNA/gel clean-up kit (GE) as per the manufacturer's instructions. The samples were prepared for NGS by the Biotechnology Platform of the ARC-OVR using the Nextera XT DNA Sample Preparation Kit (Illumina) according to the manufacturer's instructions. Libraries were sequenced on a MiSeq using 300 cycle version two reagent cartridges (Illumina) to produce paired end reads of approximately 150 bp each.

Consensus sequences were reconstructed using *de novo* assembly and verified against the published complete genome sequence of closely related viral strains. Sequence read quality was monitored with FastQC and cleaned to only include reads of q scores above 30. For *de novo* assembly trimmed Fastq files were processed using CLC Genomic Workbench v9.5.2 (QIAGEN Aarhus A/S). The minimum contig length was set to 1000 with all other parameters set at default. All contigs were subjected to a BLAST search to identify contigs of viral origin. Where necessary, contigs were assembled manually in Genomic Workbench v9.5.2 using the published complete genome sequence of closely related viral strains as a scaffold to guide the assembly. Consensus sequences of each passage were aligned using MEGA X (Kumar et al. 2018). The alignment was inspected manually to identify variable sites.

4. RESULTS

4.1. Viral titres on BHK adherent cells

The four FMDV samples serially passaged in BHK adherent cells were titrated and the results are shown in Table 4.1 below.

Table 4.1: Viral titres of four FMDV samples at different levels of passage on BHK21-C13 adherent cells.

Virus sample ID	**Passage 1 (1st)** (TCID50/ml)	**Passage 2** (TCID50/ml)	**Passage 3** (TCID50/ml)	**Passage 4** (TCID50/ml)	**Passage 5** (TCID50/ml)
SAT1V1	8.32	6.39	6.62	6.94	
SAT1V2	6.20	6.32	6.70	6.53	7.70
SAT1V3	6.32	7.13	7.41		
SAT1V4	7.27	6.41	7.28		

The titres for the 1st passage were notably higher for the SAT1V1 and SAT1V4 virus samples (8.32 and 7.27 $TCID_{50}$/ml respectively) but were low for SAT1V2 and SAT1V3 virus samples (6.20 and 6.32 $TCID_{50}$/ml respectively). These titres were used to calculate the amount of virus to use for the second passage. After the 1st passage on adherent cells, the goal was to reach a minimum titre of 7 $TCID_{50}$/ml at a MOI of 1:10 for use in BHK suspension cells. SAT1V3 reached this at passage 2 and it maintained this level at passage 3, SAT1V4 reached the minimum titre required at passage 3, and SAT1V2 took 5 passages to reach this level. SAT1V1 did not reach the minimum titre but came sufficiently close at passage 4 to continue to suspension culture.

4.2. Viral titres on BHK suspension cells

Samples generated during the three passages in suspension cells of each virus were titrated and the titres were compared for the three passages. The results are shown in the graphs below.

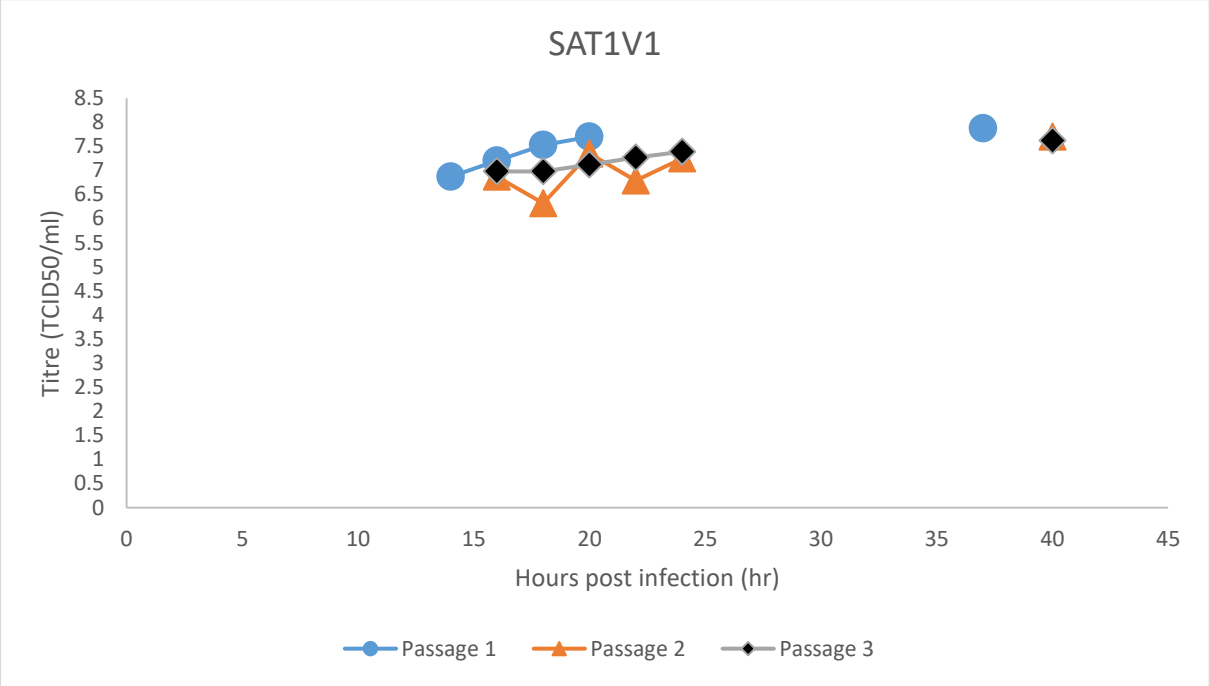

Figure 4.1 SAT1V1 viral titres of samples taken at different hours post-infection during three virus passages on BHK21-C13 suspension cells. Blue circle data points represent the 1st passage level. Orange triangle data points represent the 2nd passage level and black rectangle data points represent the 3rd passage level.

Figure 4.1 shows the titres of the samples generated during the three passages for the SAT1V1 virus sample. During the 1st passage (Figure 4.1 blue circles data points) the titres increased with increasing hours post infection (hpi) as expected, from 6.87 $TCID_{50}$/ml at 14 hpi to 7.87 $TCID_{50}$/ml at 37 hpi. The titres for the second passage (Figure 4.1 orange triangles data points) showed inconsistency and were lower than those of the 1st passage. The titres for the 3rd passage (Figure 4.1 black rectangles data points) were slightly lower than that of the 1st passage but higher in the initial hpi than the second passage, although the values converged at 34 hpi.

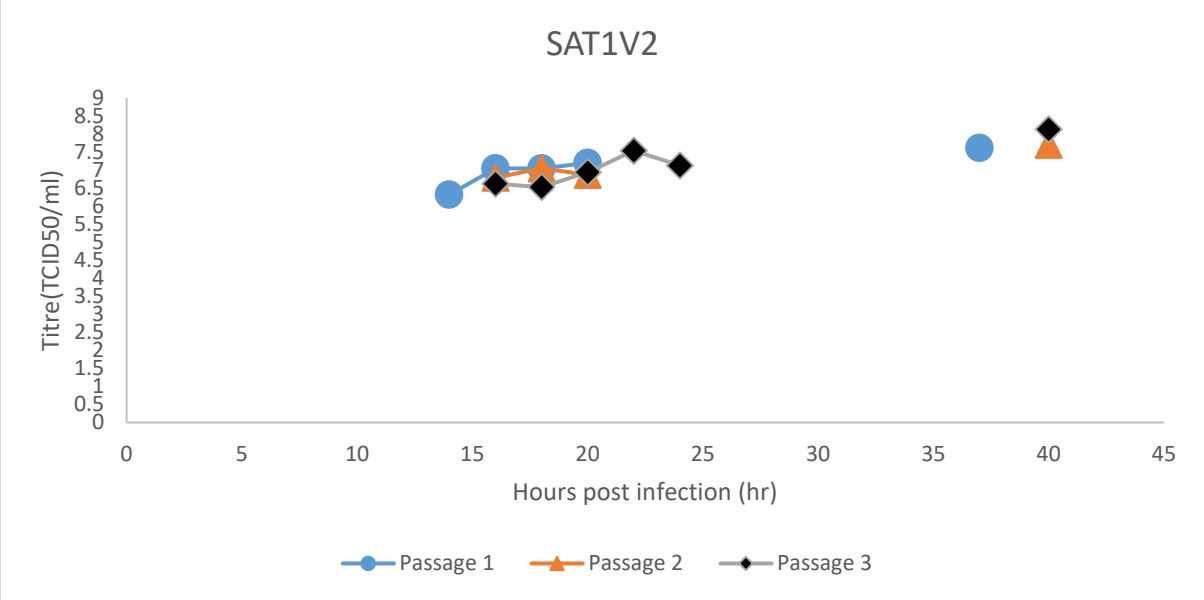

Figure 4.2 SAT1V2 viral titres of samples taken at different hours post-infection during three virus passages on BHK21-C13 suspension cells. Blue circle data points represent the 1st passage level. Orange triangle data points represent the 2nd passage level and black rectangle data points represent the 3rd passage level.

Figure 4.2 shows the titres of the samples generated during the three passages for the SAT1V2 virus sample. During the 1st passage (Figure 4.2 blue circles data points) the titres increased with increasing hpi as expected. The titres for the second passage (Figure 4.2 orange triangles data points) were slightly lower than those of the 1st passage. The titres for the 3rd passage (Figure 4.2 black rectangles data points and line) also increased with an increase in hpi.

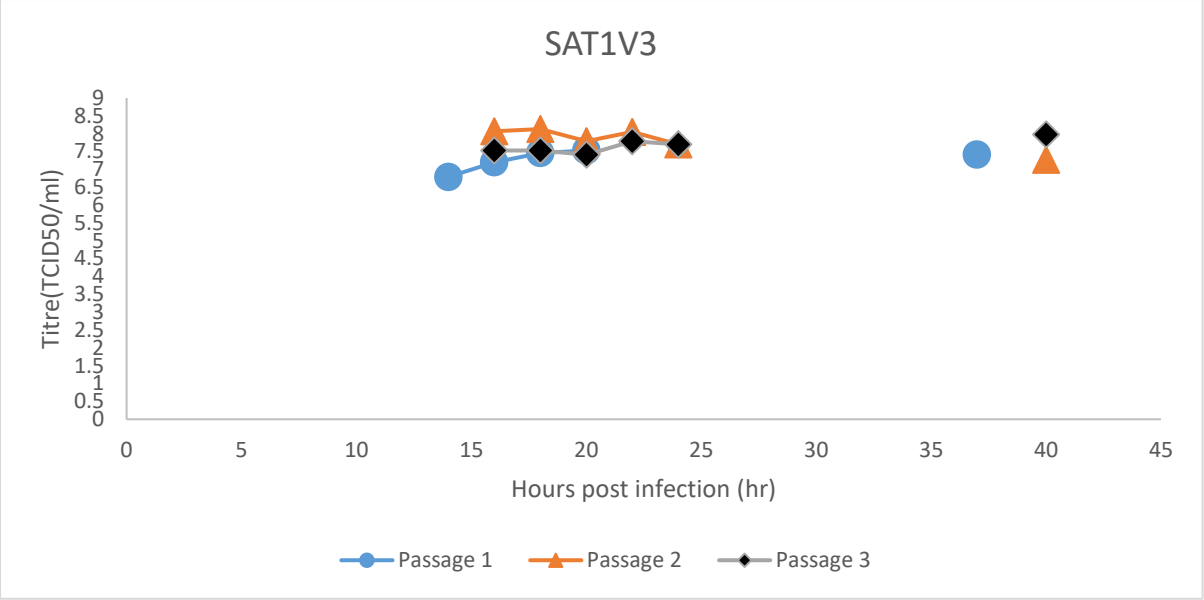

Figure 4.3 SAT1V3 viral titres of samples taken at different hours post-infection during three virus passages on BHK21-C13 suspension cells. Blue circle data points represent the 1st passage level. Orange triangle data points represent the 2nd passage level and black rectangle data points represent the 3rd passage level.

Figure 4.3 shows the titres of the samples generated during the three passages for the SAT1V3 virus sample. During the first passage (Figure 4.3 blue circles data points) the titre increased between 16 and 20 hpi, and decreased to the 16 hpi level at 37 hpi. The titres for the second passage (Figure 4.3 orange triangles data points) were higher than those of the 1st passage at 18 hpi, returned to the 16-hour level at 22 hpi and decreased between the 22nd and 40th hpi. The titres for the 3rd passage (Figure 4.3 black rectangles data points) were slightly higher than those of the 1st passage but lower than those of the 2nd passage.,

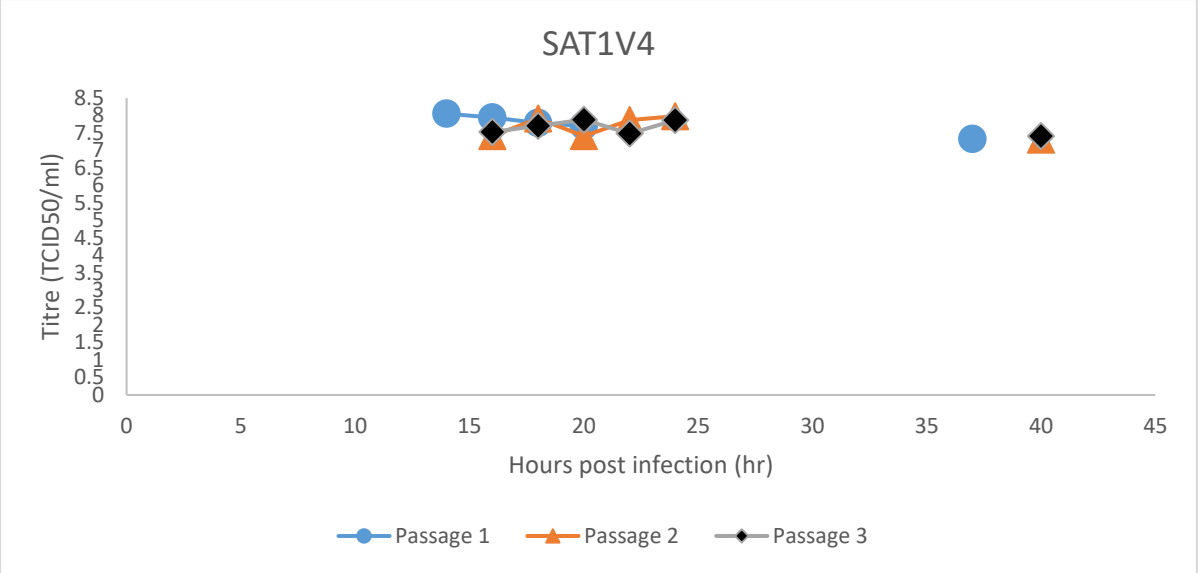

Figure 4.4 SAT1V4 viral titres of samples taken at different hours post-infection during three virus passages on BHK21-C13 suspension cells. Blue circle data points represent the 1st passage level. Orange triangle data points represent the 2nd passage level and black rectangle data points represent the 3rd passage level. .

Figure 4.4 shows the titres of the samples generated during the three passages for the SAT1V4 virus sample. During the 1st passage (Figure 4.4 blue circles data points) the titres decreased with increasing hpi between the 14th and 37th hour sampling points. The titres for the second passage (Figure 4.4 orange triangles data points) increased between 16- and 18-hour post infection, decreased between 18- and 20-hours post-infection, increased again between 20 and 24 and decreased again between 24 and 40 hours post infection. The titres for the 3rd passage (Figure 4.4 black rectangles data points) increased with increasing hour post-infection with the exception of one data point at 22 hpi and they look like they maintained this increase.

4.3. Next Generation Sequencing of viral RNA

NGS generates a large numbers of short read sequences that can be assembled into a consensus sequence representing the complete genome of the target virus. Here we used NGS to reconstruct the genomes of FMD viruses harvested from sequential passages on suspension cell cultures. Viral RNA extracted from the original virus and passages 1 and 3, of all FMD SAT1 viruses were submitted for NGS. Unfortunately, the data recovered from one or more of the libraries for SAT1V1, SAT1V2, and SAT1V3 were of low quality and could not be used to assemble the consensus sequence for all three virus samples. The effect that multiple passages has on the genetic stability of the FMD was therefore only assessed for SAT1V4.

The libraries for each of the passages of SATV4 yielded 3,382,676 (original virus), 5,142,788 (passage 1) and 4,750,420 (passage 3) sequence pairs. Each of these data sets were used to construct contigs using *de novo* sequence assembly. Table 4.2 summarises the contig measurements for each of the passages of SAT1V4 after *de novo* assembly. The contigs were analysed using BLAST to identify which contigs correspond to the viral genome. For the first virus sample (original virus) of SAT1V4 a single contig of three contigs larger that 1000 base pairs aligned to the complete genome sequence of FMDV isolate SAT1/NAM01/2010 (KU821590;(Van Borm et al., 2016). Assembly of the first and third passages yielded a single contig of 7593 base pairs that aligned to the same reference sequence, with the library sequences for passage 3 assembling into five contigs larger than 1000 base pairs that were of viral origin. The contigs for the original virus and the two passages were assembled into a single consensus sequence manually using the complete genome of FMD SAT1/NAM01/2010 as a guide (figure 8.1, appendix 1).

Table 4.2. A summary of the contig measurements for each of the passages of SAT1V4 after de novo assembly of the paired-end sequence libraries.

	SAT1V4 original virus	SAT1V4 Passage 1	SAT1V4 Passage 3
N75	383	400	824
N50	446	490	1,095
N25	544	709	1,735
Minimum	200	200	501
Maximum	18,112	14,039	14,041
Average	472	514	1,102
Count	9,516	128,513	10,425
Total	4,493,199	66,043,316	11,491,539

The consensus sequences were of unequal length preventing the comparison of the nucleotide sequences across the entire genome. The coverage of the sequence assembly was lower at the 5′ and 3′ termini of genomes sequenced. This was expected due to the recognised difficulty of recovering reliable sequences from the ends of RNA molecules. The consensus sequences were trimmed down to the coding region of the full translated polyprotein resulting in three consensus sequences of 7017 base pairs representing the FMD viral populations for each of the three samples. The aligned consensus sequences were analysed to identify any single nucleotide variants. Only one change was detected in the consensus sequence of the third passage suspension culture virus, which resulted in a non-synonymous amino acid change 38 amino acids upstream of the RGD. This substitution arose from a third base position mutation (C → A) which results in the replacement of asparagine (which has a polar side chain) with lysine (which has a positively charged side chain) (figure 9.1, appendix 2). The consensus sequences of the virus from the two earlier passages were identical across the entire polyprotein coding region of the FMDV genome.

5. DISCUSSION

In this research project, the adaptation of four low-passage FMDV SAT type virus to both BHK-monolayer and suspension cells was investigated. The results from this study direct the selection of best candidate vaccine strains that would ultimately guide the selection of the virus isolate(s) that is (are) best-suited to vaccine production in BHK-21 suspension cell cultures.

Mowat and Chapman in 1962, demonstrated that BHK21 clone 13 monolayer was capable of replicating FMDV (Mowat and Chapman, 1962), therefore the first approach was to take the four viruses that were isolated on IB-RS cells and inoculate them on BHK21 clone 13 monolayer. The four viruses performed differently on the first passage on BHK21 clone 13 monolayer. SAT1V1 and SAT1V4 had high titres above 7 TCID50/ml which suggested that the strength of infectivity to the BHK21 monolayer was high at first, whereas SAT1V2 and SAT1V3 had titres below 7 TCID50/ml and low infectivity. To adapt and stabilise the viruses, more passages were needed and the minimum titre required was 7 $TCID_{50}$/ml which is the current standard used for antigen production (ARC-OVR FMD vaccine production). The titre for the 1st passage was the baseline for subsequent passages, and they were used to calculate the amount of virus to ensure that all four viruses were infected at the same MOI. At the same MOI, only the SAT1V3 reached the minimum titre at second passage and stabilised suggesting the adaptation took less time, whereas the others viruses took more passages and thus longer to adapt.

For large-scale vaccine production, BHK21 cells adapted to grow in suspension are preferred over monolayer as the closed system eliminates the “multiple open operation” associated with adhesive cell thus BHK21 cells. In addition, virus can be grown in suspension in large volumes in bioreactors (Doel, 2003). This system is also currently used at ARC-OVR for vaccine production. For production purposes, adapted strains would need to yield enough virus over a short period of time. The current virus vaccine strains have incubation times from 8 to 16 hours at MOI of 1:10. Attaining the same high yield within a short period of time is therefore a requirement for each of the four candidate vaccine viruses, and was assessed by regular monitoring on suspension cells over three passages.

Looking at 14 hours post infection which was chosen as a starting point for sampling for the 1st passage on suspension cells, one virus (SAT1V4) was above the minimum titre of 7 TCID50/ml, the other three were below. At 16 hours post infection, all the viruses seem to reach the minimum titre and they maintained this going to the 20th hour post-infection suggesting stability. However, none of the viruses reached convincing levels of virus yield. All four viruses were then passaged for the second and third time in suspension cells. The expectation was that titres for the third passage would be higher than the 1st passage. SAT1V3 and SAT1V4 were closest to this expectation when the window of 14-22 hours was considered, with both having virus titres above the 7 $TCID_{50}$/ml level and reaching ~8 at 22 and 24 hpi, respectively, whereas SAT1V1 and SAT1V2 had lower virus titres, sometimes even lower than that of the 1st passage, between 16 and 22 hours post infection. Despite these differences, all the viruses seem stable in this cell system but of the four viruses assessed SAT1V3 followed by SAT1V4 were the best options based on yield and speed of adaptation to suspension cells cultures at 2nd and 3rd passage.

As much as the speed of adaptation and antigen yields are important for vaccine production purposes, genetic stability of the virus is also important. Studies have shown that genetic mutations acquired during adaptation promote the use of primary and secondary receptors, therefore adaptation to cell culture (Dill and Eschbaumer, 2020). However, genetic mutation in major antigenic sites may result to antigenic drift (Anil et al., 2012). The NGS was performed to compare the genetic variation, if any, introduced between the original viruses (low passage on IB-RS), and the first and the third passages on suspension cell cultures. The NGS results were not of sufficient quality to allow for comparisons for the SAT1V1, SAT1V2 and SAT1V3 virus samples. However, full-length genomes were generated for the IB-RS2 seed virus of SAT1V4 and for the first passage of the virus on suspension cell culture and the consensus sequence whilst shorter than the others for the 3rd passage sample, was sufficiently long to allow for comparison of a 7017 nucleotide region corresponding to the full-length polyprotein sequence. The sequences of all three viruses were therefore trimmed prior to analysis to ensure that the three consensus sequences were the same length. A BLAST nucleotide search of the SAT1V4 IB-RS2 trimmed consensus sequence against the Genbank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed this virus to be most closely related to FMDV isolate SAT1/NAM01/2010

(97% nucleotide sequence identity). Alignment of the three consensus sequences confirmed that the 1st passage virus on suspension cells was identical to the starting low passage IB-RS2 seed virus. In contrast, the 3rd passage virus differed from the two earlier passage viruses at a single nucleotide site; C to A substitution at polyprotein nucleotide position 2502. This third-base position mutation results in a non-synonymous mutation; Asparagine (N) replaced with positively-charged Lysine (K) at amino acid position 111 in the F-H loop of VP1. This N111K substitution upstream of the RGD motif has been previously been identified as a key mutation in SAT-1 virus adaptation to cell culture that correlates with the ability of the virus to use glycosaminoglycan molecules for cell entry (Maree et al. 2010). These results suggest that SAT1V4 is highly stable and has only undergone a single genetic change induced by the adaptation of the virus to suspension cultures and associated with associated the ability to use an alternative cell receptor. These preliminary results suggest that SAT1V4 is a genetically stable virus with moderate speed of adaptation and adequate virus yield, and one that warrants further investigation for vaccine candidacy potential.

6. CONCLUSION

In this study, adaptation of four different SAT 1 viruses in BHK21 cell line was performed to examine their attributes as possible vaccine strains. These attributes were based on speed of adaptation, virus yield and virus stability. An attempt to correlate the speed of adaptation with changes in variant composition and antigen yield was precluded by suboptimal NGS sequencing results for three of the four viruses evaluated. It was shown that the speed of adaptation to BHK21 cell cultures varied within the four FMD virus isolates, that virus yield increased with increasing time post-infection up until 24 hours, but not necessarily between passages as anticipated. The NGS results confirmed that SAT1V4 is a highly stable virus that displays minimal genetic changes within the polyprotein consensus sequence in response to adaptation. This, together with moderate levels of adaptation and virus yield make this a vaccine candidate strain that warrants further investigation. It is unfortunate that the other 3 virus strains were not subjected to NGS analysis, therefore advisable that they also warrant further investigation.

Adaptation to cell cultures of FMD field isolates can be time consuming and in a large scale vaccine production environment, very expensive. The knowledge gained from these adaptation studies together with genetic variation studies when combined with virus or cell line engineering and immunological studies will promote faster adaptation and higher yields, within a shorter space of time. Ultimately leading to more affordable vaccines.

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Annexure 1

Animal ethics committee approval certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

1 March 2021

Approval Certificate
New Application

AEC Reference No.:	REC132-20
Title:	Selection and adaptation of South African Territories (SAT) 1 and 2 foot-and-mouth disease virus for vaccine production in Southern Africa
Researcher:	Mr T Veto
Student's Supervisor:	Prof AD Bastos

Dear Mr T Veto,

The **New Application** as supported by documents received between 2020-10-01 and 2021-02-05 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2021-02-05.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number**
Cattle (Bovine)	
Samples Retrospective/stored cell cultures	4

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2022-03-01.
3. Please remember to use your protocol number (REC132-20) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadinuwa

Annexure 2

Section 20 approval letter

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform & Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Mr Henry Gololo • Tel: +27 12 319 7642 • Fax: +27 12 319 7470 • E-mail: HenryG@daff.gov.za
Reference: 12/11/1/1 (1639 LJvR)

Thando Veto
ARC-OVR TAD
100 Old Soutpans Road
Onderstepoort
Email: VetoT@arc.agric.za; HealthL@arc.agric.za;

Dear Thando,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Your application sent per email on 2 September 2020, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The study is approved as per the application form sent 2 September 2020 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HenryG@daff.gov.za;
3. All potentially infectious material utilised, collected or generated during the study are to be destroyed at the completion of the study. Records must be kept for five years for auditing purposes;
4. Only Foot and mouth disease viruses stored in the National FMD virus bank at ARC-OVR may be used;
5. These viruses can be adapted in baby hamster kidney cell lines from ARC-OVR in the BSL3 Transboundary Animal Diseases Laboratory;

Annexure 3
ARC-AEC

AEC 19:14

APPROVED

ARC • LNR

Submission Date	2019-02-01	For Administrative Purposes AEC REF.	19:14
AEC approval Date	2019-02-14	Signature (only on approval)	

A. PROJECT NO:
P10 000067

B. PROJECT TITLE
Selection and adaptation of South African territory 2 (SAT 2) foot-and-mouth disease virus for vaccine production in south Africa.

C. PROJECT LEADER/RESEARCHER (PRINCIPAL INVESTIGATOR)

Name	Contact Number	e-mail address	Contact Address
Mr Thando Velo	0720287036	velot@arc.agric.za	TAD/OVR, ARC Old Soutpan Rd, Onderstepoort
Qualifications		BSc, BSc (Hon),	
Appropriate experience in animal research		Experience in foot-and-mouth disease vaccine production	
Details of involvement		Running of experiments for the adaptation of virus strain and analysis of results.	

D. RESEARCH TEAM MANAGER (RTM)

Name	Contact Number	e-mail address	Contact Address
Dr. Livio Heath	012 529 9601	HeathL@arc.agric.za	TAD/OVR, ARC Old Soutpan Rd, Onderstepoort
Division	ARC-OVR (TAD-Campus)		
Qualifications	BSc, MSc, PhD		
Appropriate experience in animal research	Experience in animal experimentation involving ASF and FMD vaccines for vaccine efficacy trials and antisera production.		
Signature:	Date: 31/01/2019		

E. RESPONSIBLE VETERINARIAN

Name	Contact Number	e-mail address	Contact Address
N/A			
Division			

- 2 -

8. Appendix 1

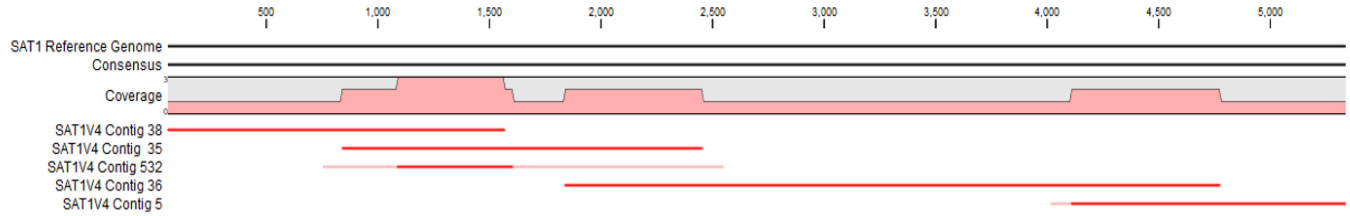

Figure 8.1 an example of the assembly of the contigs for SAT 1 V4.

9. Appendix 2

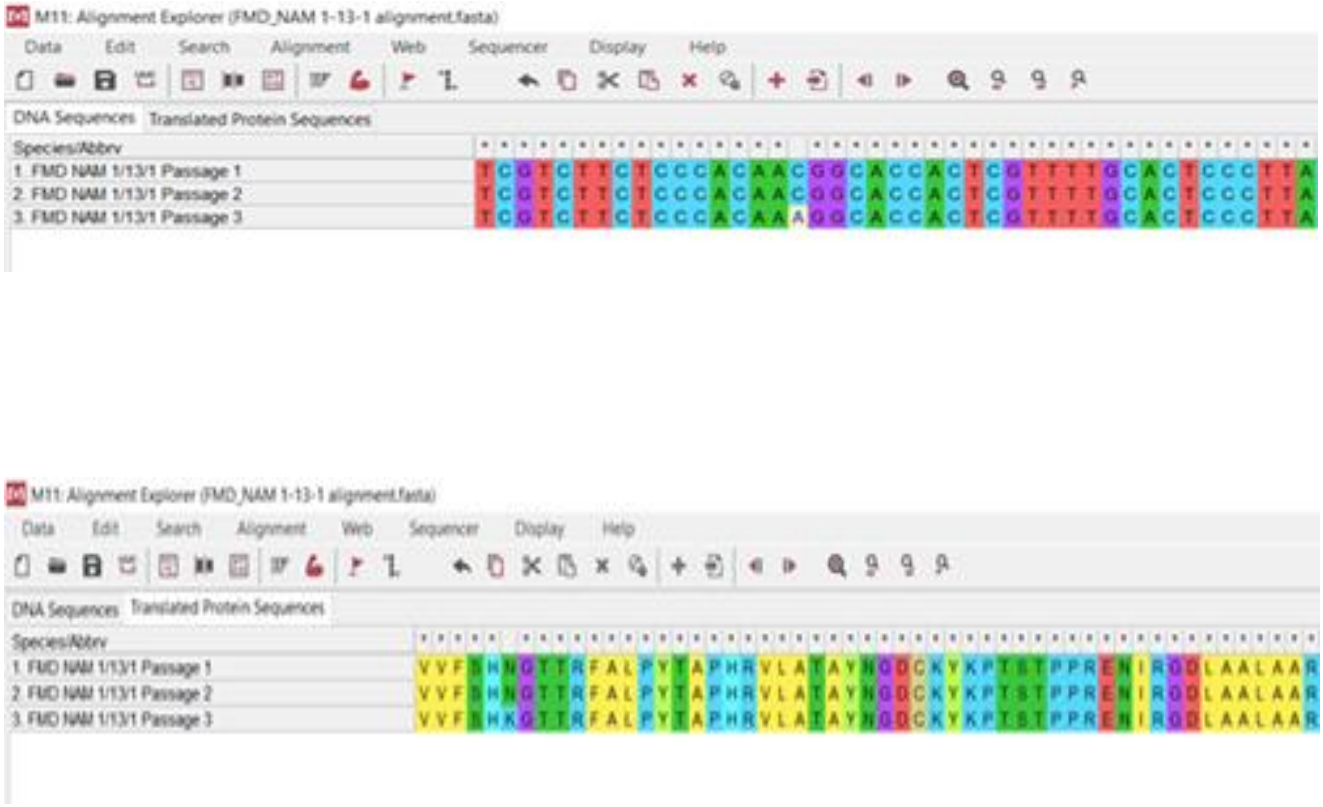

Figure 9.1 nucleotide and amino acid alignment of the three virus passages showing the nucleotide substitution C → A resulting to amino acid change from asparagine to lysine.